

Phytochemical and Pharmacological Investigation of the Methanolic Extract of *Commelina communis* (Whole Plant): Cytotoxic Potential, Analgesic Effect, and Phenolic–Flavonoid Contents

Md. Mahbubol Alam^{1,2,*}, Suraiya Binta Kader³, Nowrin Rashid Ratri³, Eity Akter³, Durdana Abthahi³, Faria Afrin³ & Fahamida Binta Anower^{1,3}

¹Department of Pharmacy, Faculty of Life and Earth Sciences, Jagannath University (JnU), Dhaka, Bangladesh.

²Department of Pharmacy, Faculty of Science and Engineering, University of Information Technology and Sciences (UITS), Dhaka-1212, Bangladesh.

³Department of Pharmacy, Faculty of Science, Engineering and Technology, Bangladesh University (BU), Dhaka, Bangladesh.

Corresponding Author (Md. Mahbubol Alam) Email: mahbubhasan089@gmail.com*



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ABSTRACT

Background: *Commelina communis* is a medicinal herb which has been used for the treatment of different disorders and contains various bioactive phytoconstituents. The current study was designed to assess the cytotoxic and analgesic properties of the methanolic extract of *Commelina communis* and to determine its total phenolic and flavonoid contents.

Methods: Cytotoxic activity was screened using the brine shrimp lethality bioassay using vincristine sulfate as a standard. Analgesic activity was evaluated using the acetic acid-induced writhing model in Swiss albino mice. The total phenolic content (TPC) and total flavonoid content (TFC) were estimated using gallic acid and quercetin standards, respectively.

Results: In the brine shrimp lethality assay, the methanolic extract was found to possess concentration dependent cytotoxicity leading to 80% mortality at the highest concentration tested, while vincristine sulfate caused 100% mortality at higher concentrations. In the analgesic test, at a dose of 500 mg/kg, the extract significantly reduced the number of writhes from 44 in the control group to 24, thereby showing 45.45% inhibition, while diclofenac sodium (100 mg/kg) inhibited the writhing response by 90.91%. The results of the phytochemical screening suggested that the extract was rich in phenolic and flavonoid contents, where TPC and TFC of the extract was 14.94 mg GAE/g extract and 149.22 mg QE/g extract, respectively.

Conclusion: The methanolic extract of *Commelina communis* possessed significant cytotoxicity, moderate analgesic activity and significant phenolic and flavonoid contents. Thus, the current results suggest that *Commelina communis* can be considered as a promising source of bioactive phytochemicals and needs further investigation for isolation and pharmacological studies.

Keywords: Analgesic Activity; Brine Shrimp Lethality; *Commelina communis*; Cytotoxicity; Methanolic Extract; Phenolic Compounds; Flavonoids; Phytochemical Analysis.

1.0. Introduction

Plants have always been one of the major sources of medicine and still remain significant in discovering new drugs. According to WHO, a considerable number of people all over the world use traditional herbal medicine as their main means of treatment [1]. Natural products extracted from plants are known to be highly rich in secondary metabolites such as phenolics, flavonoids, alkaloids, tannins, and terpenoids, which possess different biological activities, such as antioxidant, antimicrobial, anti-inflammatory, analgesic, and anticancer effects. In other words, research work on medicinal plants is highly important in terms of discovering new substances with pharmaceutical activity [2].

Among medicinal plants, there is *Commelina communis* L. (family: Commelinaceae), or Asiatic dayflower. This plant represents an annual herb species found in Asia, in such countries as Bangladesh, China, Korea and Japan. It has traditionally been used in order to treat fever, inflammation, sore throat, edema, and some other infectious diseases. It was previously established that this plant is rich in biologically active substances, such as flavonoids, phenolic acids, polysaccharides and others. Some researchers found the extracts from this plant to possess some biological effects, namely antioxidant, anti-inflammatory, antimicrobial and protective ones [3].

Cytotoxicity test is an integral part of the initial screening of plant extracts with some pharmacological activities. Brine shrimp lethality test is a very convenient, fast, and cheap way of testing the cytotoxicity of natural products. Some positive correlation has been discovered between the brine shrimp lethality and pharmacological activities such as antitumor, pesticidal, and antimicrobial. This is the reason why this test is often used as an initial screening test for finding out prospective plant extracts [4].

Pain is one of the most common symptoms found in many pathological conditions and represents a public health problem across the world. However, even though synthetic analgesics such as NSAIDs prove to be effective for pain relief, their long-term usage causes a number of side effects, among which are irritation of the gastrointestinal tract, renal and cardiovascular disorders. That is why a lot of attention is currently paid to the study of plant analgesic agents, whose possible safety profile would be better. An acetic acid-induced writhing model in mice is the commonly applied experimental technique of evaluation of peripheral analgesic activity and is widely used in order to assess the effect of extracts from medicinal plants [5].

Phenolic and flavonoids are some of the most important groups of secondary plant metabolites. It is known that these compounds are powerful antioxidants and are able to regulate various biological processes related to inflammation, oxidative stress and damages. Determination of the total phenolic and total flavonoid contents of any plant material can be very helpful in assessment of the phytochemical profile and biological efficacy of medicinal plants. In general, high contents of these components in any medicinal plants may indicate higher biological activity of these plants and may manifest itself in different pharmacological effects, such as cytoprotective and analgesic activity [6,7].

The current research was conducted to assess the biological and phytochemical properties of the methanolic extract of *Commelina communis*. The cytotoxic activity of the studied sample was tested by means of the brine shrimp lethality assay, analgesic activity was determined by the use of the acetic acid-induced writhing test in Swiss albino mice and the total phenolic and flavonoid contents were determined with the use of the standardized colorimetric assays. The results obtained in this investigation can help in scientific substantiation of the traditional uses of *Commelina communis* and development of new studies focused on isolation and identification of active components. The objectives of the current study correlate with the experimental results presented in this paper.

1.1. Study Objectives

This current study was conducted to determine the biological properties and phytochemical composition of the methanolic extract of *Commelina communis*. Specifically, the aims of the study include:

- a) To determine the cytotoxicity of the methanolic extract of *Commelina communis* via brine shrimp lethality assay.
- b) To determine the analgesic property of the methanolic extract via acetic acid-induced writhing assay in Swiss albino mice.
- c) To determine the total phenolic content (TPC) of the extract via calibration curve of gallic acid.
- d) To determine the total flavonoid content (TFC) of the extract via calibration curve of quercetin.

- e) To determine the correlation between the phytochemical composition and biological properties of the methanolic extract of *Commelina communis*.
- f) To scientifically support the use of *Commelina communis* as a medicinal plant.

2.0. Literature Review

Search for new cytotoxic agents from natural sources still is an urgent problem in pharmaceutical industry. Natural products are known to be a source of many anticancer drugs, such as vincristine, vinblastine, paclitaxel, and camptothecin derivatives. Therefore, preliminary cytotoxic screening of potential drug candidates is an integral part of pharmacology. Brine shrimp lethality test is one of the most widespread screening techniques for evaluation of cytotoxicity of a compound. It is inexpensive, quick and reliable, as well as shows good correlation with antitumor, pestidal and antimicrobial activities. The efficiency of this method in screening of plant extracts and natural products was first shown by Meyer et al. (1982). Extracts which demonstrate lethal effects on brine shrimp nauplii are usually considered to be potential candidates for further research in anticancer and toxicology [4]. There is a number of phytochemicals, which are frequently found in medicinal plants, especially in flavonoids and phenols, which cause apoptotic and cytostatic effects and oxidative stress response [3]. In this regard, evaluation of cytotoxicity of *Commelina communis* can be highly informative.

Despite the existence of many synthetic analgesics, pain control continues to be one of the major problems in medicine. NSAIDs and opioids reduce pain effectively but are accompanied by side effects, including ulceration of the stomach, nephrotoxicity, tolerance, and dependence. Hence, there are growing interests in finding safer analgesic drugs using medicinal plants [8]. The acetic acid-induced writhing test has been widely used to investigate peripheral analgesia in laboratory animals. Acetic acid injection into peritoneum stimulates the production of endogenous substances such as prostaglandins, bradykinin, and cytokines leading to the development of abdominal constrictions or writhing reactions. The plant extract which can decrease the amount of writhes is said to have analgesic action as it inhibits inflammatory mediators [5].

Earlier research has shown that flavonoids and phenolic compounds are capable of producing analgesic effect via modulating cyclooxygenase pathway, inhibiting inflammatory mediators, and antioxidative action. That is why medicinal plants with high levels of those compounds usually reveal analgesic activity in experimental studies [5]. Phenolics are one of the major categories of plant secondary metabolites known for their antioxidant activity. The antioxidative capacity of the phenols lies in the fact that they can provide hydrogen atoms and/or electrons that help neutralize the reactive oxygen species (ROS). The ROS has been shown to cause various diseases, such as cancer, heart diseases, diabetes, and others [3].

There are many ways to determine the level of TPC. One of them is the Folin-Ciocalteu colorimetric method. The amount of phenolic compounds in an extract is usually calculated in terms of gallic acid equivalents (GAE). There is plenty of evidence about a direct link between high levels of phenols and enhanced antioxidant and pharmacological activities [6]. Moreover, phenolic compounds are known to possess cytotoxic and analgesic activities as they can regulate cellular signaling pathways, decrease oxidative stress, and suppress inflammatory

processes. Therefore, TPC quantification is a significant step in phytochemical research of the medicinal plants [5]. It is known that flavonoids are one of the most investigated phytochemicals due to their numerous biological activities. These are naturally occurring polyphenolic compounds found in fruit, vegetables, herbs, and medicinal plants. There is plenty of literature data about their antioxidant, anti-inflammatory, antimicrobial, antiviral, cardioprotective, neuroprotective, and anticancer properties [3]. The total flavonoid content (TFC) of an extract can be determined by the aluminum chloride colorimetric method. It is usually expressed in quercetin equivalents (QE). The biological effects of flavonoids can be attributed to various mechanisms of action, which include their ability to scavenge free radicals, to inhibit lipid peroxidation, to modulate the enzyme activity, and regulate inflammatory mediators. Flavonoids were shown to possess the ability to initiate apoptosis in cancer cells, suppress tumorigenesis, and reduce inflammation-induced pain response. Thus, the determination of TFC is an important part of phytochemical study intended for identification of biologically active plant extracts [7].

A number of studies have been conducted about phytochemical analysis and biological activities of *Commelina communis*. It was proven that this plant contains appreciable amounts of flavonoids, phenolic acids, polysaccharides, and other bioactive secondary metabolites with various biological activities. The therapeutic properties of *Commelina communis* are associated with the ability of the plant to act as a scavenger of free radicals, decrease inflammatory response and protect biological tissues against the damage due to oxidative stress [9]. However, reports related to the simultaneous assessment of the cytotoxicity, analgesic potential, and quantitative composition of phytochemicals in the extracts of *Commelina communis* are relatively few. Thus, more research should be done to prove the medicinal properties of the plant and reveal the active metabolites responsible for biological activity. This study is one more step towards the better understanding of biological properties of *Commelina communis* since it provides the results concerning the cytotoxic activity, analgesic effect, and the content of phenolic and flavonoid compounds in its extract.

3.0. Materials and Methods

3.1. Chemicals and Reagents

The solvent for extraction of the plant was methanol. Vincristine sulfate was employed as the reference standard in the brine shrimp lethality assay, whereas diclofenac sodium acted as the standard drug in the analgesic test. Gallic acid and quercetin were the standards for the determination of TPC and TFC, respectively. All other chemicals and reagents used in the experiment were of analytical grade.

3.2. Experimental Animals

Male and female Swiss albino mice (*Mus musculus*), weighing between 25-35 gm and aged 6–8 weeks, were employed for the analgesic activity study. The animals were obtained from an approved animal breeding laboratory. The mice were obtained from Jahangirnagar University, Savar, Dhaka, Bangladesh, and housed in standard polypropylene cages under controlled environmental conditions at a temperature of $25 \pm 2^{\circ}\text{C}$, relative humidity of 55–65%, and a 12 h light/12 h dark cycle. The animals were provided food and water ad libitum. One week prior to commencement of experiments, the animals were acclimatized to laboratory conditions. All procedures carried out in this experiment followed all ethical guidelines for laboratory animals [5].

3.3. Brine Shrimp Nauplii

The eggs of *Artemia salina* (brine shrimp) were purchased from Fish Aquarium, Katabon, Dhaka, Bangladesh. Artificial seawater was made by dissolving 38 g of sea salt in 1 L of distilled water and mixing the solution properly to make sure that the salt had completely dissolved [10].

3.4. Preparation of Crude Extract (Cold Extraction Method)

Whole plant of *Commelina communis* were collected from the Kurigram district in the spring season and identified by an experienced botanist. The collected plant were carefully washed in distilled water to get rid of dust and other contaminating materials before being shade dried at room temperature until constant weight was attained. The dried plant material was ground into coarse powder by use of a mechanical grinder. About 250g of powdered plant material was extracted by soaking in 1000 ml of methanol (solvent-to-plant ratio was 4:1) in a clean, dry glass container. The container was properly sealed and left for 12 days at room temperature for extraction and shaken occasionally. The resultant mixture was first filtered through a clean cotton cloth and thereafter through Whatman No. 1 filter paper. The filtrate was concentrated under reduced pressure by rotary evaporation to yield the crude methanolic extract. The methanolic extraction of 250 g of dried whole plant powder yielded 33 g of crude extract, corresponding to a percentage yield of 13.2% (w/w). The obtained extract was dried and stored in a clean glass container at 4°C [11].

3.5. Lethal Brine Shrimp Bioassay

In order to evaluate the cytotoxicity potential of the methanolic extract of *Commelina communis*, brine shrimp lethality bioassay test was carried out according to the procedure of Meyer et al [4]. The eggs of the brine shrimps were incubated in artificial seawater at a temperature of 25-30°C with continuous aeration and light illumination for 24-48 hours so that they hatch out. The active nauplii were separated and used for lethality bioassay to test the cytotoxicity of the methanolic extract of *Commelina communis*. Serial dilutions were made to prepare various concentrations of the extract ranging from 0.078 to 160 µg/mL. Different concentrations of the extracts were prepared and 10 live nauplii were transferred to each of the test tubes. Vincristine sulfate was used as the positive control while the artificial seawater served as the negative control. After 24 hours of incubation, the number of surviving nauplii was determined and the percentage mortality was calculated using the formula below.

$$\text{Mortality (\%)} = \frac{\text{Number of dead nauplii}}{\text{Total number of nauplii}} \times 100 \quad (1)$$

The LC₅₀ was calculated based on the percentage mortality vs. log of concentration graph [12].

3.6. Analgesic activity

The writhing activity test was performed in mice using acetic acid in order to evaluate the potential analgesic effects of the plant extracts. Five mice were selected in each of the three randomly chosen groups. Group one was considered the control group while 10 mL per kg of body weight of distilled water was used. The drug Diclofenac Sodium was used in group two in a dosage of 100 mg/kg body weight. The dosage of the plant extract used in group three was 500 mg/kg body weight. The experimental extract, drug and the control substance were administered

orally 30 minutes prior to intraperitoneally injecting 0.7% v/v acetic acid. After five minutes, the number of writhes was counted after 15 minutes [13]. The percent writhing inhibition was then determined by comparing the results obtained from the experimental and standard groups to the writhing mean of the control group using this equation:

$$\text{Writhing inhibition (\%)} = 1 - \frac{\text{Writhing mean of control}}{\text{Writhing mean of sample/standard}} \times 100 \quad (2)$$

3.7. Quantification of Total Phenolic Content (TPC)

Determination of the Total Phenolic Content (TPC) was done using the Folin-Ciocalteu colorimetric assay with gallic acid as the reference substance. In brief, 0.5 mL of the plant extract solution (1 mg/mL) was reacted with 2.5 mL of 10% (v/v) of the Folin-Ciocalteu reagent. After 5 minutes of incubation, 2.0 mL of 7.5% (w/v) sodium carbonate solution was added to the reaction mixture. The mixture was vortexed well and left in the dark for 30 minutes at room temperature. The absorbance of the resultant blue-colored mixture was then measured using UV-Visible spectrophotometer at 765 nm using the reagent blank. The calibration curve was constructed by using different concentrations of the gallic acid standard solutions, and the TPC was recorded as milligrams of gallic acid equivalents per gram of the dry extract (mg GAE/g extract) [6].

3.8. Quantification of Total Flavonoid Content (TFC)

The determination of TFC was carried out by the aluminum chloride colorimetry method using quercetin as the reference substance. In brief, 0.5 mL of the extract (1 mg/mL) from the test plants was added to 1.5 mL of methanol, 0.1 mL of 10% (w/v) aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. After mixing, the sample was allowed to stand at room temperature for 30 minutes. Finally, the absorbance of the yellow-colored complex formed after incubation was measured using the UV-visible spectrophotometer at 415 nm, where a blank was run simultaneously using the reagents alone. The calibration curve was plotted using the different concentrations of quercetin standards, and the TFC was calculated in terms of quercetin equivalent (mg/g of extract) [7].

3.9. Statistical Analysis

Descriptive statistics was used to express the experimental results of phytochemical analyses (TPC and TFC). All experiments were carried out in triplicates (n = 3) and results were prepared using Excel sheet.

4.0. Result and Discussion

Cytotoxicity effect, analgesic potential, Total Phenolic Content and Total Flavonoid Content of the methanolic extract of *Commelina communis* were investigated in the present study and the findings revealed that the extract contains interesting pharmacological properties and can be considered for further research for therapeutic application as well as its traditional medicinal uses.

4.1. Cytotoxic Activity

In this study, the cytotoxic ability of the methanolic extract of *Commelina communis* were evaluated and presented in Table 1 and illustrated in Figure 1. The results indicated that the methanolic extract of *Commelina communis* has

considerable biological activity based on its dose-dependent cytotoxic activity in the brine shrimp lethality bioassay, which is one of the methods used in the primary screening of the cytotoxicity of plant extracts. In the current study, there was an increasing dose dependent mortality, calculated by Equation 1, of brine shrimp nauplii up to 80% in the highest concentration used. On the other hand, the control vincristine sulfate caused complete mortality in high doses. There was a demonstration of cytotoxic activity in the methanolic extract of *Commelina communis*. This implies that the extract contains biologically active compounds that cause interference with the cell viability process. Although it was less effective in comparison with vincristine sulfate, there is a need to perform more studies involving cell line and separation of bioactive compounds. It is suggested that the activity in the present study might be ascribed to the presence of phenolics and flavonoids due to their previously reported cytotoxic/antiproliferative effects.

Table 1. Brine Shrimp Lethality Bioassay of Methanolic Extract of *Commelina communis* (Vincristine Sulfate as Standard).

Concentration (µg/mL)	Log C	Alive Nauplii (n = 10)			Mortality (%)		LC ₅₀ (µg/mL)
		Control	Standard	Extract	Standard	Extract	
0.078	-1.108	10	9	-	10	-	Vincristine Sulfate Standard (1.07)
0.156	-0.807	10	8	-	20	-	
0.313	-0.504	10	7	-	30	-	
0.625	-0.204	10	6	-	40	-	
1.25	0.097	10	5	-	50	-	
2.50	0.398	10	3	10	70	0	<i>Stephania japonica</i> Extract (1.67)
5	0.699	10	2	9	80	10	
10	1.000	10	1	8	90	20	
20	1.301	10	0	7	100	30	
40	1.602	10	0	6	100	40	
80	1.903	10	0	4	100	60	
160	2.204	10	0	2	100	80	

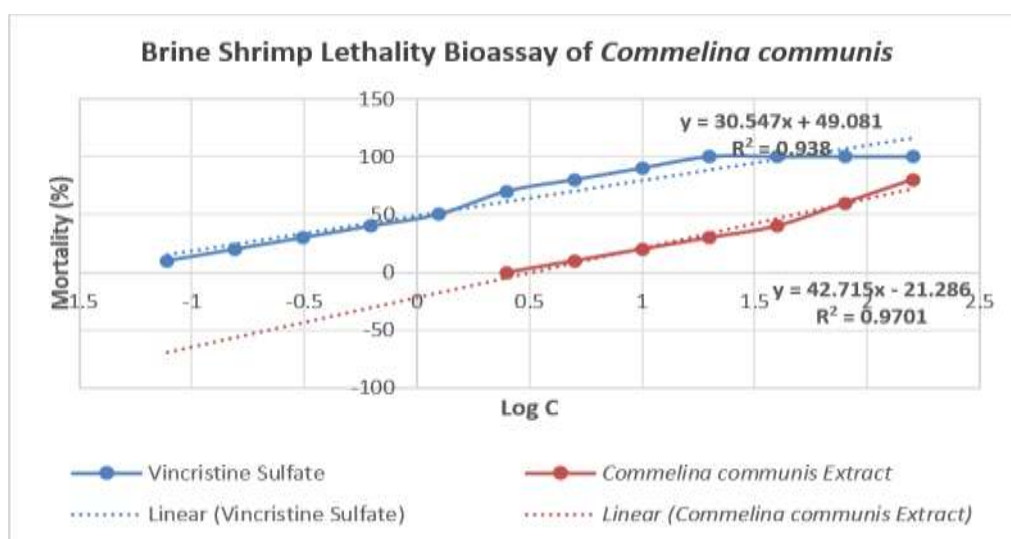


Figure 1. Comparative Percentage Mortality by Methanolic Extract of *Commelina communis* and Vincristine Sulfate.

4.2. Analgesic Activity

In the evaluation of analgesic activity, presented in Table 2 and illustrated in Figure 2, of the methanolic extract using acetic acid-induced writhing test, there was a significant reduction in writhing count in the extract treatment group as compared to the control group. Inhibition of writhing count, calculated by Equation 2, in the methanolic extract treatment group was observed to be 45.45% while the drug diclofenac sodium had 90.91% inhibition. Writhing induced by acetic acid is mediated by the liberation of various endogenous mediators including prostaglandins, bradykinin, among others in the peritoneal cavity. Hence, a decrease in writhing count is an indication of the capability of the extract to inhibit the peripheral pain mechanism. Although the extract had less analgesic potency as compared to diclofenac sodium, the significant decrease in writhing count is an indication of the existence of substances that can inhibit inflammation pathway. The analgesic action of the extract could be due to the presence of phenolic and flavonoid compounds found in it that are capable of inhibiting the formation and release of mediators of pain.

Table 2. Effect of the Methanolic Extract of *Commelina communis* on Acetic Acid-Induced Writhing in Swiss Albino Mice (n = 5).

Group	Dose	Total Number of Writhing	% Writhing Inhibition
Control	10 mL/kg	44	0
Standard (Diclofenac Na)	100 mg/kg	04	90.91
<i>Commelina communis</i> Extract	500 mg/kg	24	45.45

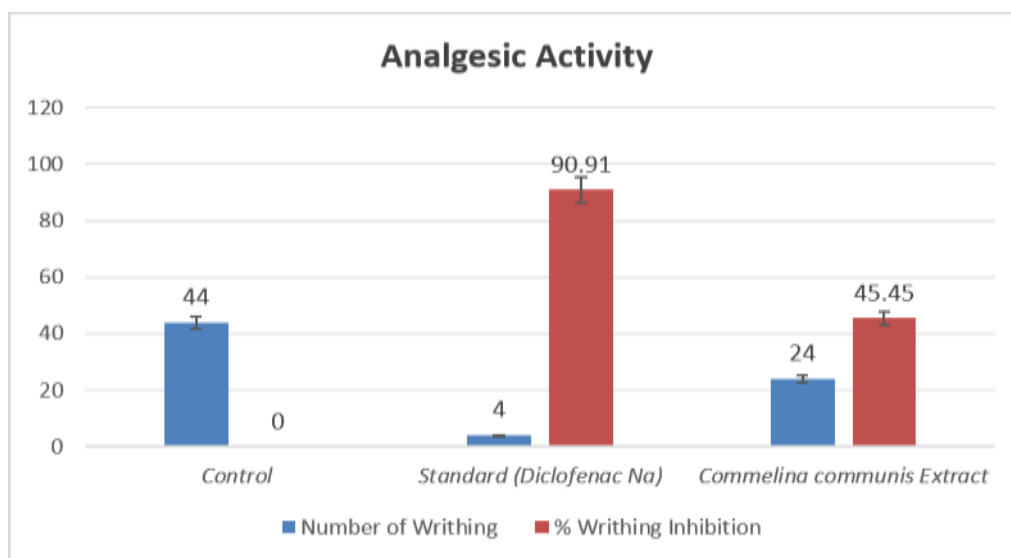


Figure 2. Analgesic Activity of the Methanolic Extract of *Commelina communis* in the Acetic Acid-Induced Writhing Test.

4.3. Quantification of Total Phenolic Content and Total Flavonoid Content

The presence of phenolic and flavonoid substances, presented in Table 3, in the methanolic extract was evident from the phytochemical screening carried out on the extract. Total phenolic and total flavonoid contents were estimated by the equation of the standard calibration curves, which are presented in Figure 3 and Figure 4, as 14.94

mg GAE/g extract and 149.22 mg QE/g extract, respectively. Phenolic and flavonoids substances are some of the most important secondary metabolites that have great roles in the biological activities of medicinal plants. They are antioxidants and are able to scavenge reactive oxygen species, reduce oxidative stress, and mediate inflammatory pathways. The high amount of flavonoids in the current work may explain partially cytotoxic and analgesic actions of the extract.

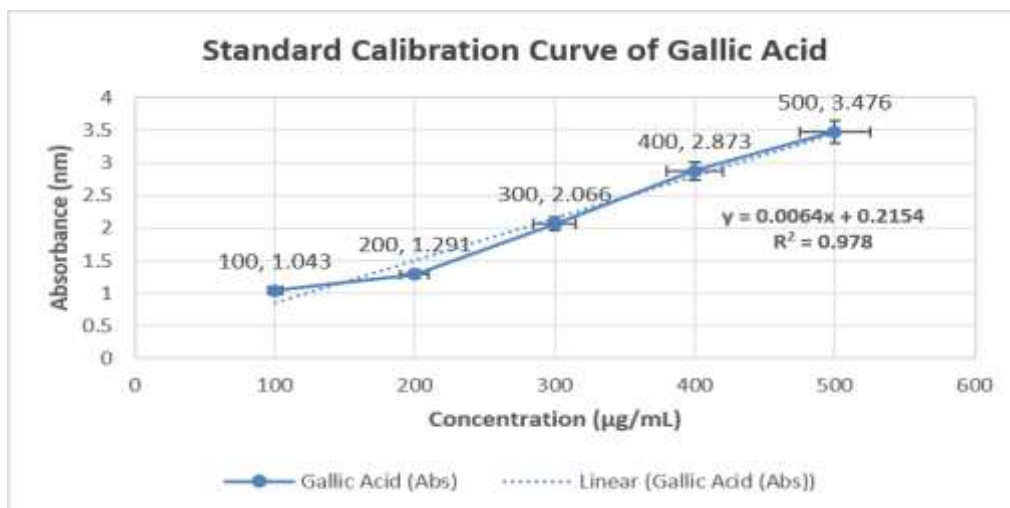


Figure 3. Gallic Acid Calibration Curve for the Determination of Total Phenolic Content in the Methanolic Extract of *Commelina communis*.

Table 3. Total Phenolic and total Flavonoid Contents of the Methanolic Extract of *Commelina communis* Extract (n=3).

Total Flavonoid Contents		Total Phenolic Content	
Absorbance (nm) Mean ± SD	Concentration (mg QE/gm extract) Mean ± SD	Absorbance (nm) Mean ± SD	Concentration (mg GAE/gm extract) Mean ± SD
0.032 ± 0.002	149.22 ± 5.09	0.311 ± 0.003	14.94 ± 0.47

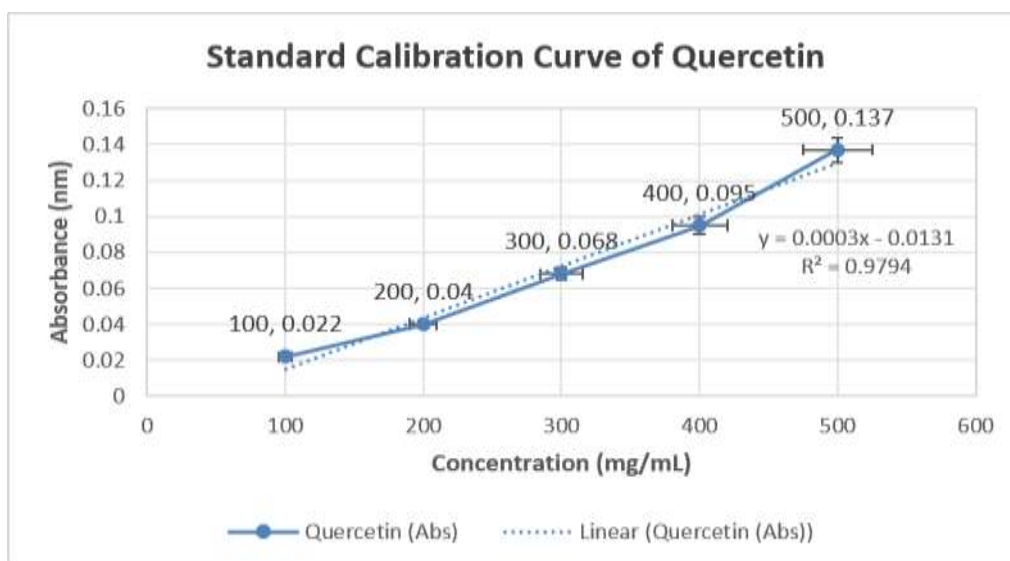


Figure 4. Quercetin Calibration Curve for the Determination of Total Flavonoid Content in the Methanolic Extract of *Commelina communis*.

The association of phytochemical composition with biological activity is one of the key elements of study concerning medicinal plants. The presence of phenolic and flavonoid constituents alongside cytotoxic and analgesic effects implies the contribution of these phytochemicals in some extent into the activity exhibited by the plant extract. Nevertheless, as the crude extract contains a mixture of various substances, it is not possible to state unequivocally which chemical compound causes this biological effect. As a result, more phytochemical studies should be conducted using chromatography for isolating the active chemicals.

4.4. Overall Pharmacological Significance

In general, based on the outcomes of the experiments on brine shrimp lethality, analgesia, and phytochemical analysis, it can be stated that the methanolic extract of *Commelina communis* has great biological potential. In particular, the extract possessed great cytotoxic activity, decent analgesic effects, and relatively high content of phenolic and flavonoid substances. Thus, it can be claimed that the scientific value of *Commelina communis* was confirmed.

5.0. Conclusion and Further Studies

5.1. Conclusion

It can be seen from the current research that the methanolic extract of the plant *Commelina communis* exhibits interesting biological and phytochemical properties. According to the results of the brine shrimp lethality bioassay, the cytotoxic activity of the extract is concentration-dependent and shows the presence of biologically active substances. Besides, moderate analgesic activity was revealed according to the results of the acetic acid-induced writhing test; the plant extracts inhibited writhing of 45.45% while diclofenac sodium inhibited writhing of 90.91%. Moreover, it was found out that the methanolic extract contains a considerable amount of the total phenolic content (14.94 mg GAE/g extract) and total flavonoid content (149.22 mg QE/g extract). Thus, the obtained results show that the methanolic extract of the *Commelina communis* plant has medicinal properties and can be considered a potential source of bioactive compounds.

5.2. Future Perspectives

In spite of the fact that the current study proved the biological potential of the *Commelina communis*, more investigations are required to identify this potential and to estimate the possible medicinal applications of the plant. In particular, future studies should be focused on:

- a) Isolation and characterization of the bioactive compounds responsible for the observed cytotoxic and analgesic activities;
- b) Phytochemical profiling using HPLC, LC-MS, GC-MS and NMR spectroscopy methods;
- c) Antioxidant activity assessment of the extract and correlation with phenolic and flavonoid contents;
- d) Mechanism of action investigation;
- e) Evaluation of cytotoxic activity against various human cancer cell lines;

f) Acute and chronic toxicity studies.

Such investigations will promote the development of new plant-derived drugs and increase the pharmaceutical significance of *Commelina communis*.

Declaration

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This study received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Competing Interests Statement

The authors declare that they have no competing interests related to this work.

Consent for Publication

The authors declare that they consented to the publication of this study.

Authors' Contributions

Md. Mahbubol Alam, Suraiya Binta Kader, Nowrin Rashid Ratri, Eity Akter and Durdana Abthahi took part in literature review, conceptualization, methodology, investigation, analysis, and manuscript writing, editing and reviewed the manuscript. Faria Afrin, Fahamida Binta Anower analyzed data. Fahamida Binta Anower was involved in the design of the manuscript. All authors have read and approved the final manuscript.

Availability of Data and Materials

Supplementary information is available from the authors upon reasonable request.

Institutional Review Board Statement

Not applicable.

Ethical Approval

All animal experiments were conducted in accordance with the institutional guidelines for the care and use of laboratory animals. Every effort was made to ensure the humane handling of animals and to minimize pain, discomfort, and stress throughout the experimental procedures.

Informed Consent

Not applicable for this study.

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Declaration of Artificial Intelligence

This research is entirely human-made and does not use any artificial intelligence.

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